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ORIGINAL ARTICLE

Effects of hyperoxia periodic training on free radicals production, biological antioxidants potential and lactate dehydrogenase activity in the lungs of rats, *Rattus norvigicus*

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Abstract Oxygen therapy has been widely used in lung injury (Li), adult respiraotory syndrome (ARDS) and inflammatory lung diseases as well as in mechanical ventilation in intensive care units. Exposure to hyperoxia is known to induct the production of reactive oxygen species (ROS) by mitochondria. Despite decades of research, the role of hyperoxia training in oxidative stress and ROS formation in the lungs is not known. The purpose of this study was to examine the effects of periodic-hyperoxia training on biological antioxidants (BAP) and lactate dehydrogenase (LDH) activities and free radicals (FR) production. Thirty adult male rats, matched with age and body weigh, were randomly assigned to three groups. The first group served as control (C) and the second (HP) was exposed to hyperoxia for 48. Animals in the third group (HP-T) were trained on hyperoxia for 1.5 h daily for three weeks. Following the exposure period for each group animals were sacrificed and lungs tissues were homogenized for BAP, LDH and FR determinations. LDH activity was determined by Randox protocol (Randox – UK). BAP and FR were determined using dROM method (H&D – Italy). Results showed that mean (±SD) BAP activity increased significantly ($p < 0.05$) from the baseline control of 7105.88 ± 2021.49 to 8611.20 ± 1245.26 (U/L) after hyperoxia training; then dropped to 6784.00 ± 1879.50 during hyperoxia exposure for 48 h. Whereas mean (±SD) FR production increased significantly ($p < 0.05$) from the baseline control of 262.50 ± 67.52 to 339.90 ± 64.84 during HP exposure for 48 h, then dropped to 211.13 ± 52.05 (Carr), during HP training. Similarly, LDH activity increased significantly ($p < 0.05$) from the

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baseline control of 210.31 ± 70.93 to 339.90 ± 64.84 during HP exposure for 48 h, then dropped to 159.30 ± 20.61(U/L), following HP-periodic training. Furthermore, the correlation ($r = 0.67\times$) of LDH on FR was significant ($p < 0.05$), implying that reduction in ROS generation induced by HP-periodic training is related to reduced rate of cell apoptosis caused oxidative stress. Based on the results of the present study HP-periodic training is recommended in order to resist oxidative damage in the lungs.

1. Introduction

Under normal physiological and metabolic conditions, single electrons sometimes escape from the transport chain which is located in the inner mitochondrial membrane and result in a single electron reduction of molecular oxygen forming a superoxide anion (O_2^-). Oxidative mitochondrial stress (OMS) occurs when O_2 tension is increased because the buildup of O_2^- can not be controlled. Thus superoxide production can be significantly enhanced during exposure to hyperoxia because the rate of electron transport is limited by the buildup of a large proton gradient in the inner mitochondrial membrane leading to variety of mitochondrial pathological changes such as swelling, concentrated cristae, dilution of the inner and outer membrane (Haffor, 2004; Haffor and Al-Johany, 2005). Clearly both OMS and O_2^- generation lead to the production of ROS such as hydrogen peroxide (H_2O_2) which in the presence of ferrous iron via the Fenton reaction, result in highly reactive hydroxyl radicals. Once mitochondrial and cellular enzymatic biological antioxidant potential (BAP) are overwhelmed by the buildup of ROS, oxidative damage and the subsequent cell death can occur. Therefore it can be expected that long duration exposure to hyperoxia can be a potential cause for numerous common lung diseases because it can triggers OMS, pneumocyte death as well as circulating neutrophils and alveolar macrophages defense responses. These defense responses can also contribute to magnification of ROS generation. It has been shown that exposure to hyperoxia beyond 24 h result in morphologic changes that are similar to pulmonary inflammation, atelectasis, oedema formation, irreversible loss of respiratory function and lung inflammation (Crapo, 1986; Jankov et al., 2003; Jafari et al., 2004).

Lactate dehydrogenase (LDH) catalyses the terminal step in anaerobic glycolytic pathway which is located in the cell cytoplasm. The elevated level of LDH reflects high compensatory anaerobic rate secondary to oxidative mitochondrial stress (OMS). Thus LDH activity can be reliable cytosolic early marker for OMS and the subsequent elevation in ROS formation.

The effects of hyperoxia and risk of bronchopulmonary dysplasia in infants or adult respiratory distress syndrome in adults begins with exposure period over 8 h (Arieli, 1998; Chavko et al., 1998; Demchenko et al., 2001). In healthy adult risk begins after 48 h (Comroe et al., 1945). Because symptoms caused by hyperoxia, requires long duration exposure, it can be speculated that short term exposure can cause adaptive protective changes related to OMS, ROS production and antioxidants activities. The role of periodic short duration exposure to hyperoxia on ROS production and enhancement of antioxidants resistance is not known nor have been examined in previous studies. The purpose of present study was to explore the effects of periodic-hyperoxia training (PHT) on free radicals (FR) production, lactate dehydrogenase (LDH) and biological antioxidant potential (BAP) activity.

2. Materials and methods

2.1. Experimental design

Twenty-four adult Wister Albino male rats, *Rattus norvigicus*, matched with age and body weigh, were randomly assigned to four groups, ten animals each. The first group served as control and the second, was exposed to hyperoxia for 48 h. Animals of the third group were engaged in periodic-hyperoxia-training (PHT) for 1.5 h daily for 3 weeks period. The control and experimental groups were sacrificed and the lungs were isolated and homogenized immediately in 0.9 saline solutions (4:1 ratio). All animals were treated according to standards described in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by National Institute of Health (NIH Publications 86–23 revised 1985).

2.2. Hyperoxia exposure

Animals of the first experimental group were placed in a closed box that has an inlet flow which was connected to 100% O_2 tank, medical grade, on which a regulator was connected to maintain flow at 5 l per minute (LPM). The out flow of the regulator passed through a humidifier in order to saturate the inspired air with H_2O. The outlet ventilation rate of the box was adjusted at 5 LPM to ensure that the concentration of oxygen in the box remains equal to 100% O_2 and maintain normal flow and maintain normal barometric pressure at 767 mm Hg. The temperature inside the box was adjusted at room temperature (22–24 °C).

2.3. Hyperoxia periodic training

Animals of the second experimental group were engaged in hyperoxia training program for three weeks period. Exposure to hyperoxia was conducted for thee intervals, for 30 min each, separated with 10 min breathing room air, normoxia.

2.4. Free radical determination

Free radicals production was measured, using the d-ROMs-4 test kits (Health & Diagnostic, Italy) according to the manufacturer's instructions. The test measures the levels of hydroperoxides (R-OOH) which are generated by peroxidation of biological compounds; lipid, amino acids, nucleic acids. This test is based on the principle of the ability of hydrogen peroxides to generate free radicals after reacting with some transitional metals (Fe_2^+/Fe_3^+), according to Fenton's reaction as follows:

$$H_2O_2 + Fe^{2+} = {}^{*}OH + OH^- + Fe^{3+}$$

Table 1 Descriptive data for the dependent measures, FR, BAP, and LDH in the lungs of the three groups.

Animal	Control			HP-48			HP-Trained		
	FR	BAP	LDH	FR	BAP	LDH	FR	BAP	LDH
1	326.25	7312.5	297.14	401	7147	297.14	274	8124	165.08
2	191.25	4226.25	140.32	270	4155	264.13	154	7512	140.32
3	296.25	8002.5	272.38	383	8063	272.38	233	9356	173.34
4	187.5	4117.5	206.35	263	4158	206.35	146	7152	132.06
5	326.25	9461.25	247.62	360	8254	247.62	255	10,092	181.59
6	296.25	6843.75	148.57	379	5700	305.39	244	8100	148.57
7	360	10511.25	321.91	450	10,350	321.91	278	11,212	189.84
8	202.5	7616.25	132.06	285	6900	198.09	169	8124	132.06
9	180	6003.75	197.27	274	6401	272.38	154	8040	173.33
10	258.75	6963.75	139.49	334	6712	222.86	206	8400	156.83
Mean	**262.50**	**7105.88**	**210.31**	**339.90**	**6784.00**	**260.83**	**211.13**	**8611.20**	**159.30**
SD	**67.52**	**2021.49**	**70.93**	**64.84**	**1879.50**	**41.94**	**52.05**	**1245.26**	**20.61**

Thus, the hydrogen peroxides of biological sample (whole blood) generate free radicals (alcoxy and peroxyl radicals) after exposure to a transitional metal (F^{2+}/Fe^{3+}). When a correctly buffered chromogen substance (N,N-diethyl-phenylendiamine) lead to the reduction of hydrogen peroxides which in turns colored as radical cation. Color intensity was read using spectrophotometer with peak absorbance of 505 nm. In the d-ROMs test results were expressed in CARR units (CARR U). One CARR U relates to 0.08 mg H_2O_2/100 ml.

2.5. BAP test principle

The biological antioxidant potential (BAP) was measured, using the BAP-4 test kits (Health & Diagnostic, Italy) according to the manufacturer's instructions. BAP represents plasma barrier component with reduction oxidation potential that gives reducing equivalents to reactive species. BAP is based on the ability of a colored solution, containing ferric (Fe^{3+}) ions adequately bound to a special chromogenic substrate to decolor when its Fe^{3+} ions are reduced to ferrous (Fe^{2+}) ions as well as it can be observed by adding a reducing system, that is sample of blood plasma. When plasma sample is dissolved in a colored solution that is been obtained by mixing a source of ferric ions ($FeCl_3$), R2 reagent with special chromogenic substrate (sulfer-derived compound), R1 reagent. After a short period of incubation about 5 min, the solution will decolor and the intensity of this change will be directly proportional to the ability of plasma to reduce ferric ions.

2.6. LDH determination

Samples from all groups were used for the determination of LDH activities using Randox protocol (Randox, England). The principle of this method is based on the following reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \rightarrow \textit{l}\text{-lactate} + NAD^+.$$

2.7. Statistical analysis

Mean group differences for the dependent variables; free radicals (FR) and biological antioxidants potential (BAP) were evaluated using one-way analysis of variance (ANOVA) to reveal the main effect of each group on the dependent variables. Tukey–Kramer multiple comparisons were used to compare differences between each means pairs. Linear regression model was used to examine the relationship between FR and LDH.

3. Results

The mean final body weights (±SD) of the three groups; control, hyperoxia exposure for 48 h and hyperoxia periodic training; at the end of the experiment were 196.57 ± 6.41, 198.66 ± 11.39, and 193.33 ± 8.09 g, respectively. Results of paired *t*-test indicated that values were not significantly ($p > 0.05$) different than body weights prior to the experiment.

The lung tissues data for free radicals (FR), biological antioxidants (BAP) and lactate dehydrogenase (LDH) activity are presented in Table 1. Exposure to hyperoxia for 48 h continuously resulted in increasing mean (±SD) FR production in lungs from the baseline control of 262.50 ± 67.52 to 339.90 ± 64.84; then dropped to 211.13 ± 52.05 (CARR U), following hyperoxia periodic exposure for 3 × 30 min each, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period. Mean (±SD) LDH activity increased from base line control of 210 ± 31 70.93 to 260.83 ± 41.94 (U/L), following exposure to hyperoxia continuously for 48 h, then dropped to 159.30 ± 20.61 (U/L), following hyperoxia periodic exposure for 3 × 30 min each, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period. Fig. 1 displays the mean changes for LDH and FR among groups. In addition, the average (±) BAP activity in the lung increased from the baseline control of 7105.88 ± 2021.49 to 8611.20 ± 1245.26 μl/ml, following hyperoxia periodic exposure for 3 × 30 min each, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period (Fig. 2).

Results of one-way (ANOVA) analysis of variances (Table 2) showed significant ($p < 0.05$) differences among groups' means for free radicals (FR), biological antioxidants (BAP) and lactate dehydrogenase (LDH) activities. Post-hoc Tukey–Kramer multiple comparisons procedures (Table 3) were conducted to simultaneously examine comparisons between all possible pairs of group means. When hyperoxia exposure administered for 48 h, it elevated both FR and LDH activity significantly ($p < 0.05$), then dropped significantly ($p < 0.05$)

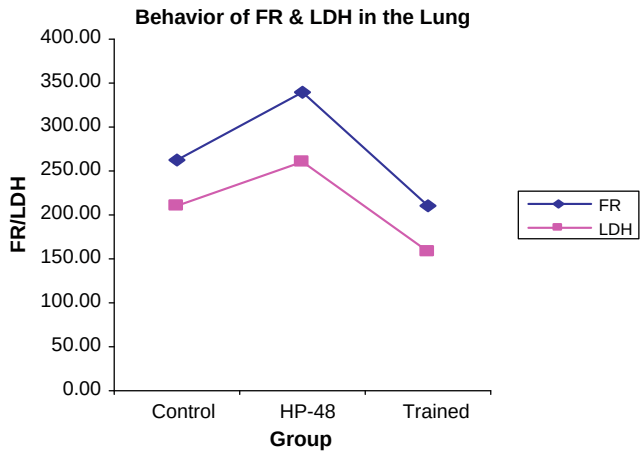

Figure 1 Mean changes for LDH and FR among groups.

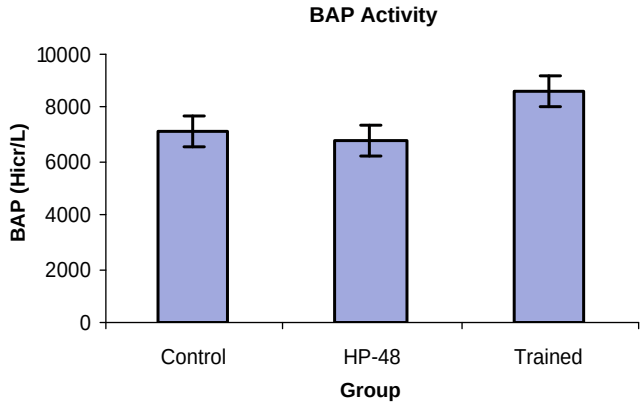

Figure 2 Mean changes for BAP among groups.

following hyperoxia periodic exposure for 3×30 min each, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period. When hyperoxia exposure administered for 48 h continuously, there was no significant ($p > 0.05$) change in biological antioxidant potential (BAP), but it increased significantly ($p < 0.05$) Following hyperoxia periodic exposure for 3×30 min each, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period. The regression of LDH on FR (Fig. 3a) after hyperoxia interval exposure for 3×30 min each, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period was significant ($p < 0.05$), $r = 0.67$ (Fig. 2), with significant ($p < 0.05$) regression sum of squares (Table 4). This regression model was validated using residual sum of squares, which did not contain pattern and was symmetrically distributed around the zero line (Fig. 3b).

4. Discussion

Hyperoxia is believed to generate reactive oxygen species (ROS) and inhibit antioxidants defense. O_2 toxicity is believed to occur when the body's antioxidant defenses are overwhelmed by increased production of ROS. Included in ROS list are superoxide, hydrogen peroxide, hydroxyl radicals, and peroxynitrite at high levels of $PtiO_2$ (Demchenko et al., 2001; Demchenko et al., 2003; Torbati et al., 1992). Herein, the present study showed that biological antioxidant potential (BAP) in the lungs was overwhelmed following 48 h of continuous hyperoxia exposure.

It is clear that dysfunctional mitochondria result in releasing its contents such as oxidative enzymes and hydrogen peroxides to the cytoplasm, in attempt to prevent swelling. When the rate of release of mitochondrial contents exceeds elimination rate by antioxidants defense system, ROS accumulate and FR productions rise. Previous work showed that hyperoxia inducted variety of pathological changes in the inner mitochondrial membrane (Haffor, 2004) that provided critical mitochondrial events responsible for oxidative stress-mediated cell death known as toxic oxidative stress (TOS). Herein the present study showed that hyperoxia exposure for 48 h resulted in an increase in free radicals production associated with reduction in biological antioxidant potential (BAP).

The severity of hyperoxic-induced cellular injury is time- and dose-dependent (Hayatdavoudi et al., 1981; Barry and Crapo, 1985; Crapo et al., 1994). The effects of hyperoxia and associated risk of bronchopulmonary dysplasia in infants or in adult respiratory distress syndrome (ARDS) in adults begins with exposure period over 8 h (Arieli, 1998; Chavko et al., 1998a; Demchenko et al., 2001. In healthy adult risk begins after 48 h (Comroe et al., 1945). However, acute exposure to hyperbaric-hyperoxia causes tissue and cellular damages in the brain as early as 16 h of exposure (Huang et al., 2000; Gerstner et al., 2006). Besides lung epithelial ROS generation, capillary endothelial cells were identified as the source of hyperoxia-induced ROS production (Kuebler et al., 2000).

Table 2 One-way ANOVA for the dependent variable.

S. variation		Sum of squares	*df*	Mean square	*F*	Sig.
FR in lung	Between groups	83833.867	2	41916.933	10.951*	.000
	Within groups	103349.375	27	3827.755		
	Total	187183.242	29			
BAP in lung	Between groups	19027555.704	2	9513777.852	3.113	.061
	Within groups	82526554.631	27	3056539.060		
	Total	101554110.335	29			
LDH in Lung	Between groups	51535.006	2	25767.503	10.715*	.000
	Within groups	64928.906	27	2404.774		
	Total	116463.912	29			

* $p < 0.05$.

Table 3 Multiple comparisons – LSD.

Dependent variable	Group membership	Group membership	Mean difference	Std. error	Sig.
FR in lung	Control	HP-48	−77.40*	27.68	.009
		HP-trained	51.20	27.67	.075
BAP in Lung	Control	HP-48	321.87	781.86	.684
		HP-trained	−1505.33	781.86	.065
LDH in lung	Control	HP-48	−50.51*	21.93	.029
		HP-trained	51.01*	21.93	.028

* The mean difference is significant at the .05 level.

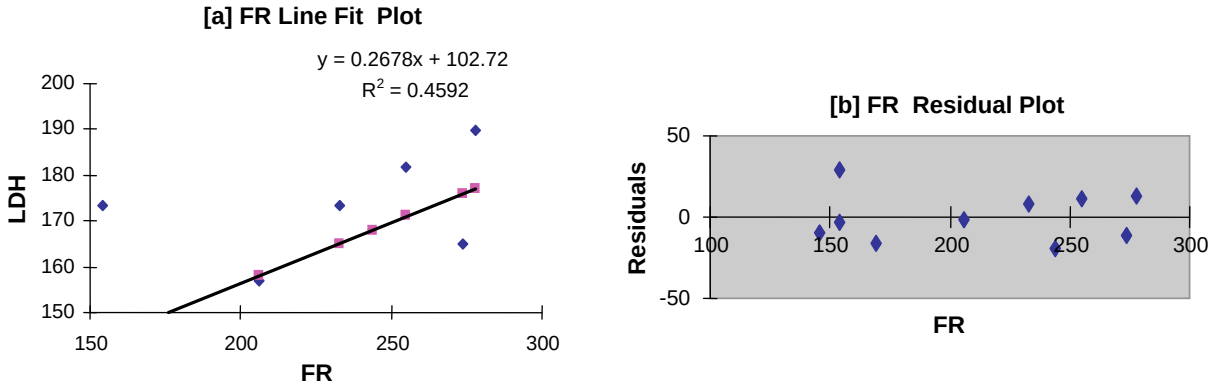

Figure 3 The regression of LDH on FR (a) after hyperoxia training, residual sum of squares (b), with no pattern.

Table 4 The regression of LDH on FR following HP training.

Regression statistics	
Multiple *R*	0.677669
R square	0.459236
Standard error	16.07415
Observations	10

ANOVA

	df	SS	MS	*F*	Significance *F*
Regression	1	1755.39	1755.39	6.79387*	0.0313
Residual	8	2067.026	258.3783		
Total	9	3822.416			

* $p < 0.05$.

Herein, results of the present study showed a steady rise in ROS generation, beyond the onset of lung tissue injury as reported in these studies, which reflected additional phagocytes defense mechanism of the alveolar macrophages which in turn contributed additively to ROS generation.

Although increased generation of ROS is evident in lung epithelia cells in vitro within 30–60 min of hyperoxia (Manautou and Carlson, 1991; Sanders et al., 1993; Parinandi et al., 2003), clinical use of normobaric hyperoxia for several hours is frequently considered harmless or even recommended to reduce the risk of post-surgical wound infections (Neubauer et al., 1994; Greif et al., 2000; Belda et al., 2005) and head injury (Brown et al., 1988).

In humans, the first respiratory symptoms have been reported after 6 h of oxygen exposure (Comroe et al., 1945), and ultrastructural alterations such as epithelial cell swelling are evident within 14 h but with hyperoxia of 70% O_2 (Kapanci et al., 1972). In rats, animals die within 60–72 h of exposure to 100% O_2, whereas an FIO_2 of 0.85 is sublethal but may cause platelet accumulation within 3 days and increase lung weight within 5 days of exposure (Crapo et al., 1980; Barry and Crapo, 1985; Tibbles and Edelsberg, 1996). Herein, results of the present study clearly showed lower rate of FR generation following periodic exposure to hyperoxia for 30 min 3×, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period.

Many clinicians have successfully reduced oxygen toxicity by the antioxidant redoxiredoxin administration (Kim et al., 2003) and selenium supplementation (Ebert et al., 2006). Others used nitro oxide to prevent cell apoptosis in the lungs

(Iben et al., 2000; Howlett et al., 1999). In the present study, BAP sustained its rise in attempt to defend the integrity of the pneumocyte following periodic exposure to hyperoxia for 30 min 3×, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period.

The continued exposure to hyperoxia and the associated continued rise in free radical production along with failure of defense mechanisms to effectively neutralize toxic intermediates acted as stimulus on LDH. It was reported that superoxide oxide radical (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) intermediates all have a high oxidizing potential and thus are responsible for cellular oxygen toxicity (Farr and Kogoma, 1991; Fridovich, 1998; Storz and Imlay, 1999). Herein, results of the present study showed an increase in LDH activity following the continued exposure to hyperoxia for 48 h, which followed similar behavior to FR production. Furthermore, it was shown that the change is glycolytic and mitochondrial enzymes affects LDH activity (Haffor and Alhazza, 2007). Herein result showed that the mitochondrial and the cytoslic regulatory mechanism for LDH were recalled as such the improvement in LDH was related to FR, following periodic exposure to hyperoxia for 30 min 3×, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period.

In conclusions: the results of the present study along with the data showing that LDH and FR levels decreased in the lungs following periodic exposure to hyperoxia for 30 min 3×, intervened with 10 min rest of normoxia breathing, 5 days weekly, for three weeks period. The elevated of ROS formation, associated with reduced BAP following hyperoxia exposure for 48 h in the lungs reflected additional mechanism related to phagocyte in the lungs.

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